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COLONY-FORMING UNITS IN DIFFUSION CHAMBERS (CFU-d) AND COLONY-FORMING UNITS IN AGAR CULTURE (CFU-c) OBTAINED FROM NORMAL HUMAN BONE MARROW: A POSSIBLE PARENT–PROGENY RELATIONSHIP

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ABSTRACT

A series of experiments was performed to elucidate the relationship between cells that form granulocytic colonies in fibrin clot diffusion chambers implanted into the peritoneum (i.p.) of irradiated mice (CFU-d) and day 7 and day 14 CFU-c which give rise to colonies after 7 and 14 days in agar cultures *in vitro*, respectively.

Normal human bone marrow cells were cultured in suspension *in vitro* or in diffusion chambers implanted into irradiated or non-irradiated mice. During these culture conditions there was an initial decrease in the number of CFU-c per culture. This was followed by an increase between day 2 and day 7 of culture. No similar increase of neutrophilic CFU-d was observed. When CFU-d, day 14 and day 7 CFU-c in normal marrow were separated by velocity sedimentation and cultured in suspension culture or in diffusion chambers for 7 days, the maximum increase of day 7 and day 14 CFU-c was observed in slowly sedimenting cell fractions which contained the majority of CFU-d. After 3 days in suspension culture, the maximum increase of day 14 CFU-c was found in fractions which also gave rise to maximum numbers of CFU-c after 7 days. However, day 7 CFU-c were found in fractions which initially contained the majority of day 14 CFU-c. No increase in CFU-d was found in fractions initially containing peak numbers of CFU-c. Between 53 and 71% of CFU-c harvested from diffusion chambers in irradiated mice or from suspension cultures were sensitive to pulse incubation with tritiated thymidine, suggesting that the cells were proliferating during these culture conditions. In diffusion chambers implanted into non-irradiated mice, however, CFU-c were found to be relatively resistant to this treatment (3–11% sensitive to tritiated thymidine). Thus marked increases in CFU-c were also observed during experimental conditions, where no significant DNA synthesis was detected. A

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reproducible time sequence of increase in CFU-c populations in culture was observed. Day 14 CFU-c and cells that gave rise to clusters on day 7 in agar increased between day 2 and day 4, whereas day 7 CFU-c increased between day 4 and day 7.

The results suggested that CFU-d gave rise to CFU-c in culture and that day 14 CFU-c were precursors of day 7 CFU-c.

Normal human bone marrow contains committed granulocyte–macrophage progenitor cells (CFU-c), functionally defined as cells that give rise to colonies in agar culture *in vitro* in response to macromolecules, termed colony-stimulating activity (CSA) (Robinson & Pike, 1970). *In vivo* culture techniques are now available for the detection of a family of precursor cells, CFU-d which form neutrophilic, eosinophilic and megakaryocytic colonies in fibrin clot diffusion chambers implanted into the peritoneal cavity (i.p.) of sublethally irradiated mice (Jacobsen, 1975; Jacobsen & Fauerholdt, 1976, 1977).

CFU-d which give rise to granulocytic colonies after 14 days in culture can be physically segregated from CFU-c by velocity sedimentation, a procedure which separates cells primarily according to size (Jacobsen, Broxmeyer & Moore, 1976). Furthermore, two CFU-c populations with different sedimentation velocity can be distinguished. One gives rise to colonies after 7 days in agar culture (day 7 CFU-c). A second population of CFU-c form colonies on day 14 of incubation *in vitro* (day 14 CFU-c) (Jacobsen *et al.*, 1977, 1978; Johnson, Dresch & Metcalf, 1977). These results suggest that granulocytic CFU-d, day 14 CFU-c and day 7 CFU-c are cells with increasing size, in that order.

The present study was designed to elucidate whether a parent–progeny relationship existed between the different compartments of granulocytic colony-forming units (CFU). Sutherland, Till & McCulloch (1971) and Iscove *et al.* (1972) have demonstrated the existence of a population of cells that can be separated from CFU-c by velocity sedimentation and is capable of giving rise to CFU-c in suspension culture. The present paper describes velocity sedimentation experiments which suggest the hypothesis that CFU-d may be identical with cells that give rise to CFU-c in culture.

MATERIALS AND METHODS

Details of the procedures have been described recently (Jacobsen *et al.*, 1978).

Normal human bone marrow

This was obtained from healthy volunteers by aspiration from the superior posterior iliac spine and mixed with heparin as an anticoagulant.

Density cut separation

Buffy coat cells from the marrow aspirates were separated by a bovine serum albumin (BSA) density cut procedure as previously described (Broxmeyer, Moore & Ralph, 1977). The collected buoyant fraction (density less than 1·070 g/cm^3) contained the vast majority of CFU (Jacobsen *et al.*, 1978).

Velocity sedimentation

Low density cells were separated by velocity sedimentation as described by Miller & Phillips (1969). Cells were allowed to sediment through a gradient of 0·4–2% BSA in phosphate-buffered saline at 4°C for 4–5 hr. Fractions of 30 ml were collected and the cells were counted and assayed for CFU or cultured in diffusion chambers or suspension cultures as described below. In some experiments, some of the fractions were pooled before inoculation into cultures.

Assays for CFU

(a) *CFU-c.* Cells were suspended in modified McCoy's 5A medium containing 10% fetal calf serum (FCS) and 0·3% Bacto-Agar (Difco) and plated on top of peripheral blood leucocyte feeder layers in 35 mm Petri dishes (Robinson & Pike, 1970). 10^5 unseparated low density cells or 2 to 4 × 10^4 cells from velocity sedimentation fractions were plated per dish. The cultures were incubated at 37°C in a humidified atmosphere of 7·5% CO_2 in air and scored for clusters (three to fifty cells per aggregate) and colonies (more than fifty cells) after 7 and 14 days of incubation. Three to five plates were scored per point. Cell aggregates were aspirated by means of Pasteur pipettes, smeared individually on microscope slides and stained with May–Grünwald–Giemsa stain for determination of cell morphology.

(b) *CFU-d.* Fibrin clot diffusion chambers (Millipore GS membranes, pore size 0·22 μm) were inoculated with cells in modified McCoy's 5A medium, 20% FCS and 0·5% human fibrinogen. Thrombin was added to induce coagulation of the fibrinogen suspension. Each chamber received 1·5 × 10^5 unseparated cells or 5 × 10^4 cells obtained after velocity sedimentation. The chambers were implanted, i.p., into 5–7 week old female Swiss mice (CD1, Charles River, Wilmington, Massachusetts, U.S.A.), two chambers into each mouse. All mice received 600 rad total body irradiation from a ^{137}Cs source 2–4 hr before chamber implantation. After 7 days, the chambers were removed, cleaned on the outer surfaces and re-implanted into newly irradiated mice for a further 7 days of culture. The cultures were harvested after a total of 14 days, stained with May–Grünwald–Giemsa stain and scored for neutrophilic and eosinophilic colonies (more than thirty cells) (Jacobsen, 1975; Jacobsen *et al.*, 1978). An average of twelve chambers were scored per point.

Kinetics of CFU in culture

Fig. 1 illustrates the design of experiments aimed to measure changes in numbers of CFU as a function of time in suspension culture *in vitro* or in diffusion chambers *in vivo*. The initial cell material consisted of unseparated low density bone marrow or fractions obtained after velocity separation of the cells. Aliquots of the cells were inoculated into fibrin clot diffusion chambers or plated in agar to assess the number of CFU on day 0, i.e. prior to culture.

(a) *Suspension culture.* Each culture tube received 2 to 10 × 10^5 cells suspended in modified McCoy's 5A medium supplemented with 15% FCS and 15% conditioned medium. The final cell concentration was 5 × 10^5 cells per ml. Conditioned medium was obtained by incubation of normal human adherent low density peripheral blood cells for 4–5 days at 37°C. After 1–10 days in suspension culture, the bone marrow cells were washed twice and aliquots were plated in agar or in fibrin clot diffusion chambers to assess CFU.

(b) *Diffusion chamber culture.* Cells were suspended in modified McCoy's 5A medium with 15% FCS and inoculated into diffusion chambers, 3 to 10 × 10^5 cells per culture. The

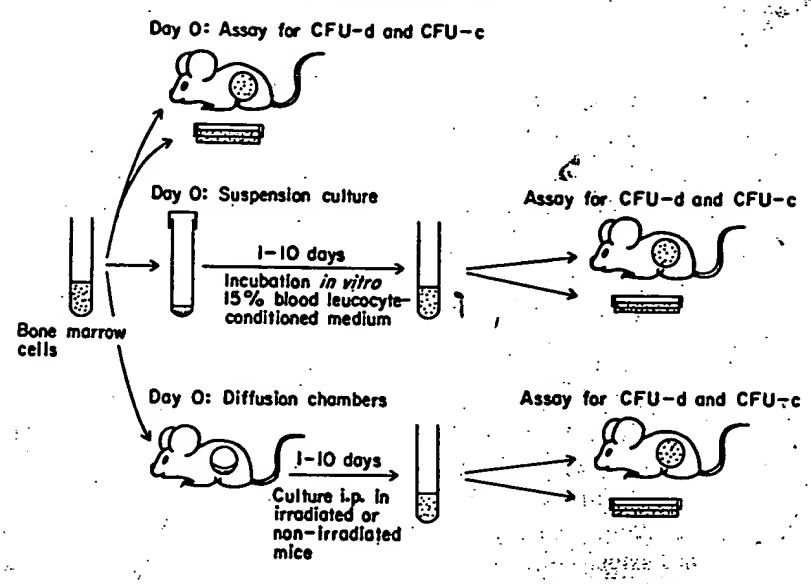

FIG. 1. Changes in numbers of CFU as a function of time in diffusion chambers and suspension culture. Low density normal human bone marrow cells (less than 1·070 g/cm³) or fractions obtained by velocity sedimentation were incubated in diffusion chambers *in vivo* or in suspension cultures *in vitro*. CFU-d and CFU-c were measured prior to culture (day 0) and at various time intervals after inoculation of the cells into cultures.

chambers were implanted i.p. into 600–900 rad ^{137}Cs-irradiated or non-irradiated mice. When the cells were cultured for more than 7 days, the chambers were re-implanted into new mice on day 7. These cultures were initially fibrin-free, but after 1–3 days a fibrin clot formed spontaneously within the chambers (Benestad, 1970). In other experiments (e.g. Fig. 4), fibrin clot diffusion chambers, identical with the cultures employed for the CFU-d assay, were used rather than initially fibrin-free chambers. The cultures were harvested after 1–10 days using a previously described technique (Benestad, 1970; Jacobsen, 1977). In short, the chambers were incubated with pronase to dissolve the fibrin clot and the cells were collected and washed three times before they were transferred to agar cultures and fibrin clot diffusion chambers for assessment of CFU-c and CFU-d, respectively.

Tritiated thymidine 'suicide' technique

To measure the proportion of CFU-c in DNA synthesis (S phase) in diffusion chambers or suspension cultures, the cells were harvested as described above. Half the cells were incubated in McCoy's medium with 15% FCS and 100 μCi per ml tritiated thymidine ([^{3}H]TdR, New England Nuclear, specific activity 20 Ci/mmol). This agent is specifically toxic to colony-forming cells in S phase (Becker *et al.*, 1965). The remaining cells were incubated in the same medium containing cold thymidine as a control. After 30 min of incubation at 37°C, the cells were washed three times in medium with cold thymidine at a concentration equal to 100 times the concentration of [^{3}H]TdR and subsequently cultured in agar to measure CFU-c. The reduction of the number of CFU-c after exposure to [^{3}H]TdR was expressed as the percentage of control CFU-c as a measure of the proportion of cells in S phase.

RESULTS

CFU kinetics in culture of unseparated marrow

Figs 2 and 3 show the results of experiments performed to obtain preliminary information about the kinetics of CFU-d and CFU-c in culture. Normal low density human bone marrow cells were incubated with leucocyte-conditioned medium in suspension cultures *in vitro* or

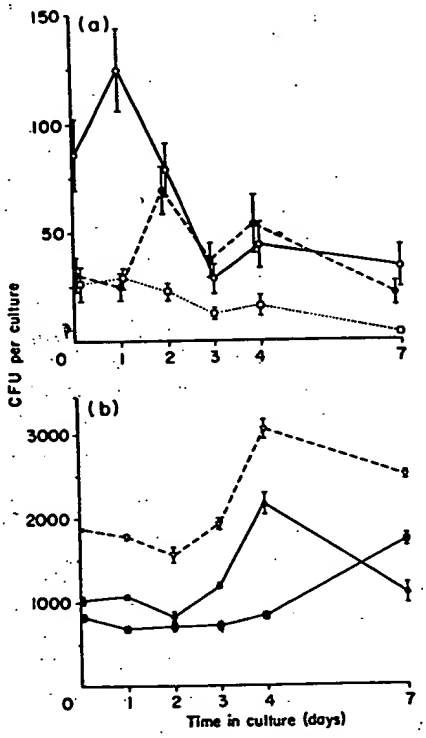

FIG. 2. Numbers of CFU as a function of time in suspension culture. Normal human low density cells cultured in suspension cultures *in vitro* with 15% adherent blood-cell-conditioned medium. Upper graph: CFU-d, capable of forming neutrophilic (○), eosinophilic (●) and megakaryocytic (□) colonies. Lower graph: CFU-c which formed clusters (▲), colonies (■) and total aggregates (▽) on day 7 in agar. All results mean ± 1 s.e.m.

inoculated into fibrin-free diffusion chambers and cultured i.p. in 900 rad irradiated or in non-irradiated mice. At various time intervals the cells were harvested, and the number of CFU-d and CFU-c per culture was assessed by further culture of the cells in fibrin clot diffusion chambers or in agar, respectively (see Fig. 1).

In suspension culture there was a decrease in the number of neutrophilic CFU-d during the first 7 days of incubation. In the experiment shown in Fig. 2, neutrophilic CFU-d increased slightly in number during the first day. In three other experiments this increase was not observed. Rather, their number was constant during the first day and then decreased. Eosinophilic CFU-d reproducibly increased and/or maintained their number throughout the culture period.

In diffusion chambers implanted into irradiated mice there was an increase in neutrophilic CFU-d to a maximum on day 2, followed by a decrease, as previously described (Jacobsen, 1977). This is illustrated in Fig. 3, which shows one of three consistent experiments. In non-irradiated mice neutrophilic CFU-d decreased in number during the first 2 days, after which time no significant changes were seen. Eosinophilic CFU-d (not shown) remained constant or increased slightly, independent of host irradiation.

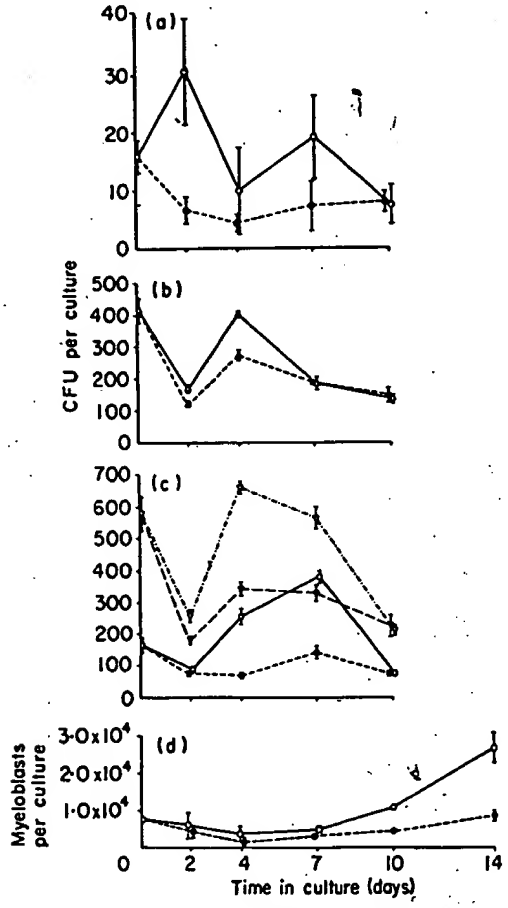

FIG. 3. Numbers of CFU as a function of time in diffusion chambers. Normal human low density bone marrow cells were inoculated into fibrin-free diffusion chambers and cultured in 900 rad irradiated or non-irradiated mice. (a) Neutrophilic CFU-d, (b) day 7 cluster-forming cells in agar, (c) day 7 total aggregate (cluster and colony) forming cells (▽, ▼) and day 7 colony-forming cells in agar (□, ■), and (d) morphologically recognizable myeloblasts are shown. All open symbols are mice irradiated with 900 rad, all closed symbols are non-irradiated mice. All results mean ±1 s.e.m.

Cells capable of forming aggregates in agar decreased initially and subsequently increased in number, both in suspension culture and in diffusion chambers (Figs 2 and 3). Cells that formed clusters on day 7 in agar increased between day 2 and day 4 of culture. Cells that formed colonies on day 7 in agar (day 7 CFU-c) increased more slowly and reached a maximum later, i.e. after approximately 7 days in culture. Morphologically recognizable myeloblasts were assessed in diffusion chamber cultures with a haemocytometer and

differential counts of cells harvested after 1–14 days of culture (Fig. 3). Increasing numbers of myeloblasts were observed on days 10–14 of culture (Bõyum *et al.*, 1972).

The recovery of CFU and myeloblasts from diffusion chambers was enhanced when the chambers were implanted into irradiated, rather than into non-irradiated mice (Fig. 3).

Origin of CFU-c in culture

To obtain information about the relationship between CFU-d and the cells that gave rise to CFU-c in culture, normal low density bone marrow cells were separated by velocity

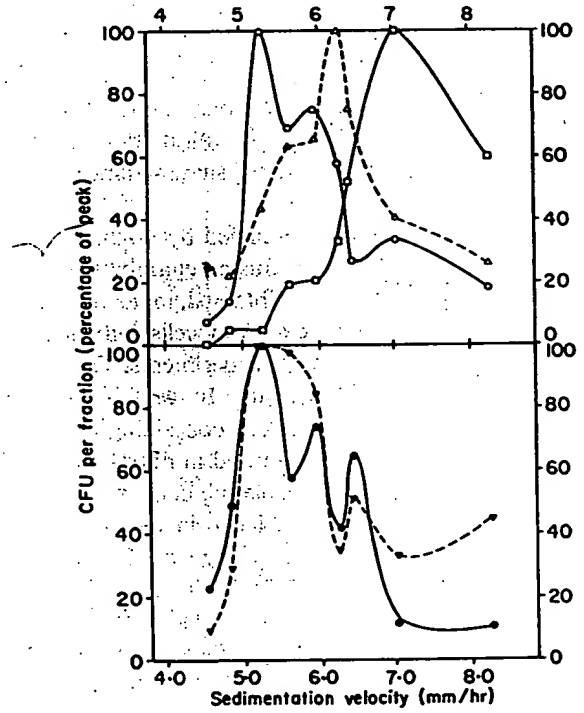

FIG. 4. Velocity sedimentation profiles of granulocytic precursor cells. Normal human low density bone marrow cells were separated by velocity sedimentation. Upper graph: neutrophilic CFU-d (○) and day 14 (△) and day 7 CFU-c (□) per fraction, as measured by fibrin clot diffusion chamber and by agar culture, respectively. Lower graph: aliquots of each fraction were cultured in fibrin clot diffusion chambers i.p. in 600 rad irradiated mice (●) and in suspension cultures in vitro (▼). After 7 days the number of CFU-c per culture was measured and the absolute yield of CFU-c per fraction was calculated. The results were normalized to a percentage of the peak number. The absolute number of CFU per fraction at the peak was: (upper graph) 357 neutrophilic CFU-d, 2608 day 14 CFU-c and 1004 day 7 CFU-c; (lower graph) 357 CFU-c recovered after 7 days in diffusion chambers, and 1122 CFU-c recovered after 7 days in suspension culture. Fractions initially containing peak numbers of CFU-d gave rise to maximum numbers of CFU-c in culture.

sedimentation, and aliquots of each fraction were inoculated into fibrin clot diffusion chambers or plated in agar to assess the initial number of CFU in each fraction. Other separated cells were incubated in suspension culture *in vitro* or cultured in fibrin clot diffusion chambers, which were implanted into irradiated mice. After 7 days, the cultures were

harvested, and the cells were plated in agar to assess the number of CFU-c yielded by each fraction in the cultures.

Fig. 4 shows the results of one such experiment. The upper graph shows the velocity sedimentation profiles of colony-forming cells, as measured immediately after separation. CFU-d were slowly sedimenting cells which formed a peak at 5·2 mm/hr. Within the CFU-c compartment two subpopulations could be segregated. Cells that formed colonies on day 7 in agar sedimented rapidly, forming a peak at 7·0 mm/hr. CFU-c that gave rise to colonies on day 14 of incubation formed a profile with peak numbers at 6·2 mm/hr, intermediate between CFU-d and day 7 CFU-c. When the cell fractions were cultured in diffusion chambers or suspension cultures and harvested after 7 days, low numbers of CFU-c were recovered in the fractions initially containing peak numbers of CFU-c. Instead, absolute increases were observed in cultures inoculated with cells that sedimented more slowly than CFU-c. Maximum numbers of CFU-c were recovered in fractions initially containing peak numbers of CFU-d. These results suggested that CFU-c in fibrin clot diffusion chambers and suspension cultures were derived from cells that had the same sedimentation velocity as CFU-d.

The lower graph in Fig. 4 shows the profiles obtained by counting agar colonies 7 days after plating the cells from suspension cultures or diffusion chambers. When the colonies were counted 14 days after plating, similar profiles were obtained, indicating that day 7 and day 14 CFU-c during these culture conditions originated from cells with identical sedimentation velocities. Similar results were obtained in four other experiments. The sedimentation velocity of CFU varied by ±10% between the experiments. In each experiment, however, the maximum increase in CFU-c was observed in fractions containing peak numbers of CFU-d. Similar profiles resulted when cell fractions were cultured in fibrin-free diffusion chambers in irradiated and non-irradiated mice for 7 days before plating in agar.

To exclude the possibility that the increase in CFU-c in cultures of slowly sedimenting fractions, or the decrease in CFU-c in rapidly sedimenting fractions, was due to the presence of interacting cells that influenced the recovery of CFU-c from the cultures, various cell fractions were mixed and co-cultured in suspensions or in diffusion chambers for 7 days. The recovery of CFU-c from such co-cultures was not significantly different from the recovery expected from separate cultures of each of the fractions.

Relationship between day 7 and day 14 CFU-c in suspension culture

The relationship between day 7 and day 14 CFU-c was studied in more details in combined velocity sedimentation and suspension culture experiments (Fig. 5). Low density bone marrow cells were separated by velocity sedimentation. Aliquots from each fraction were plated in agar immediately after separation, or were incubated in suspension culture *in vitro* for 3 and 7 days and subsequently plated in agar. Each set of agar cultures was scored for colonies both 7 and 14 days after plating. Fig. 5a shows the velocity sedimentation profiles of day 7 and day 14 CFU-c, as measured before suspension culture. Day 7 CFU-c sedimented more rapidly (peak at 6·5–7·0 mm/hr) than cells that gave rise to colonies on day 14 (peak at 5·6 mm/hr), as already described.

Fig. 5, b and c, show the profiles of cells that gave rise to day 7 and day 14 CFU-c after 3 and 7 days in suspension culture, respectively. Day 7 CFU-c, harvested after 3 days of incubation, were derived from cells which had a velocity sedimentation profile identical with the original day 14 CFU-c population (peak at 5·6 mm/hr), compatible with the contention

that day 14 CFU-c gave rise to day 7 CFU-c in suspension culture. In contrast, day 7 CFU-c, present in rapidly sedimenting fractions immediately after separation, did not give rise to detectable colony-forming cells in suspension culture. Day 14 CFU-c recovered after 3 days of suspension culture originated from a population of slowly sedimenting cells (peak at 4·7 mm/hr) which could not be identified by the profiles in Fig. 5a, i.e. before suspension culture.

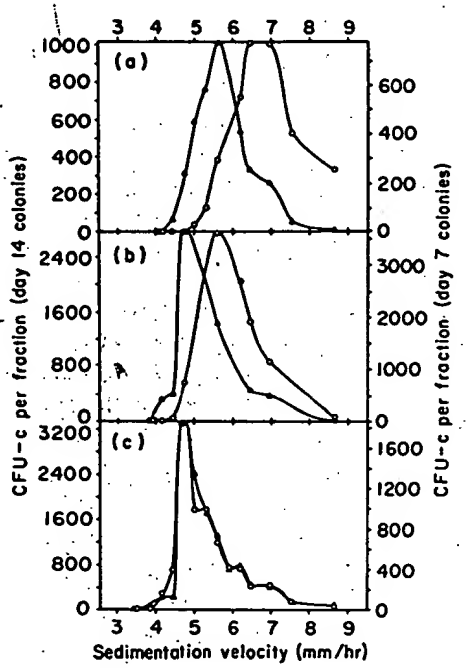

FIG. 5. Velocity sedimentation profiles of CFU-c populations. Normal human low density bone marrow cells were separated by velocity sedimentation. (a) Cells were plated in agar initially after separation to assess the number of day 14 (▲) and day 7 (○) CFU-c per fraction. (b) Fractions incubated in suspension culture for 3 days and then plated in agar, symbols as in (a). (c) Cells incubated in suspension culture for 7 days before plating in agar, symbols as in (a). The results show the absolute yield of day 7 and day 14 CFU-c per fraction. After 7 days in suspension culture (c), the maximum numbers of day 7 and day 14 CFU-c were observed in fractions of cells which sedimented more slowly than CFU-c (i.e. 4·7 mm/hr). After 3 days in suspension culture (b), the maximum numbers of day 7 CFU-c were found in fractions with an intermediate sedimentation velocity (i.e. 5·6 mm/hr), whereas day 14 CFU-c were found in fractions of slowly sedimenting cells (4·7 mm/hr).

After 7 days of suspension culture (Fig. 5c), both day 7 and day 14 CFU-c were derived from slowly sedimenting cells (4·7 mm/hr), which formed a profile identical with the profile of cells that gave rise to day 14 CFU-c after 3 days. No cell populations could be distinguished in cultures of more rapidly sedimenting cell fractions (peaks at 5·6 or 6·5–7 mm/hr).

This experiment was repeated once in full and two times using velocity fractions pooled into four or five major pools. All four experiments supported the same conclusion. Restated, these experiments suggested that the population of rapidly sedimenting day 7 CFU-c

disappeared within the first 3 days in suspension culture. Day 14 CFU-c, which had intermediate sedimentation velocities, gave rise to a population of day 7 CFU-c after 3 days, but did not give rise to detectable colony-forming cells after 7 days in suspension culture. Slowly sedimenting cells, which could not be distinguished initially, gave rise to day 14 CFU-c after 3 days and both day 7 and day 14 CFU-c after 7 days in culture.

Cycling state of CFU-c in culture

Additional information about the kinetics of CFU-c in culture was obtained by the assessment of the proportion of cells in S-phase, as measured by the [^{3}H]TdR suicide technique (Becker *et al.*, 1965). Cultures were inoculated with unseparated low density bone marrow cells (one experiment) or low density cells which sedimented at between 4·5 and 5·6 mm/hr by velocity sedimentation (three experiments). These fractions contained the majority of CFU-d but relatively low numbers of CFU-c. The cells were placed in suspension cultures or inoculated into fibrin-free diffusion chambers, which were implanted into 600 rad irradiated or non-irradiated mice. After 2, 3, 4 and 7 days the cells were harvested, exposed to [^{3}H]TdR or cold thymidine and plated in agar cultures.

Table 1 shows the mean results of the four experiments. Identical results were obtained with cultures inoculated with unseparated low density marrow cells and fractions of slowly sedimenting cells. Furthermore, the proportion of [^{3}H]TdR-sensitive cells was identical in cultures harvested on days 2, 3, 4 and 7. For this reason, the results for all days were pooled, as shown in Table 1.

TABLE 1. Proliferative state of CFU-c in culture

Colonies	Suspension culture	Diffusion chambers: Irradiated mice*	Diffusion chambers: Non-irradiated mice
Day 7 colonies	71 ± 10†	59 ± 5·0	3·1 ± 7·2
Day 7 clusters	69 ± 4	53 ± 3·8	7·3 ± 3·9
Day 14 colonies	70 ± 6	56 ± 3·3	11 ± 4·0

Cells harvested on days 2, 3, 4 and 7. Mean results from four separate experiments.

* All mice received 600 rad 2–4 hr prior to chamber implantation.

† Percentage reduction in CFU-c after exposure to [^{3}H]TdR, ±1 s.e.m.

High proportions (53–71%) of day 7 CFU-c, day 7 cluster-forming cells and day 14 CFU-c were sensitive to [^{3}H]TdR when the cells were harvested from suspension cultures or diffusion chambers in irradiated mice, suggesting that they were proliferating during these culture conditions. In contrast, no significant killing of CFU-c and cluster-forming cells was observed when the cells were cultured in diffusion chambers in non-irradiated mice, indicating that the cells were not in DNA synthesis in this type of culture. The cycling state of neutrophilic CFU-d in diffusion chambers was measured in two of the experiments. Evidence of DNA synthesis was observed in irradiated but not in non-irradiated mice, as previously reported (Jacobsen, 1977).

Kinetics of CFU in diffusion chambers in non-irradiated mice

Since the results presented in Table 1 suggested that cells within the CFU-c compartment were not proliferating in diffusion chambers in non-irradiated mice, these culture conditions

were particularly suited to study increases in CFU-c as a result of recruitment from more immature cell compartments.

Fig. 6 shows an example of a detailed study of the kinetics of CFU-d and CFU-c in this culture system. Normal low density bone marrow cells were separated by velocity sedimentation. The fractions were pooled into four major fractions, which were inoculated into fibrin-free diffusion chambers and cultured in non-irradiated mice. Chambers were harvested after 1, 2, 4 and 7 days, and the number of CFU-d and CFU-c per culture was determined.

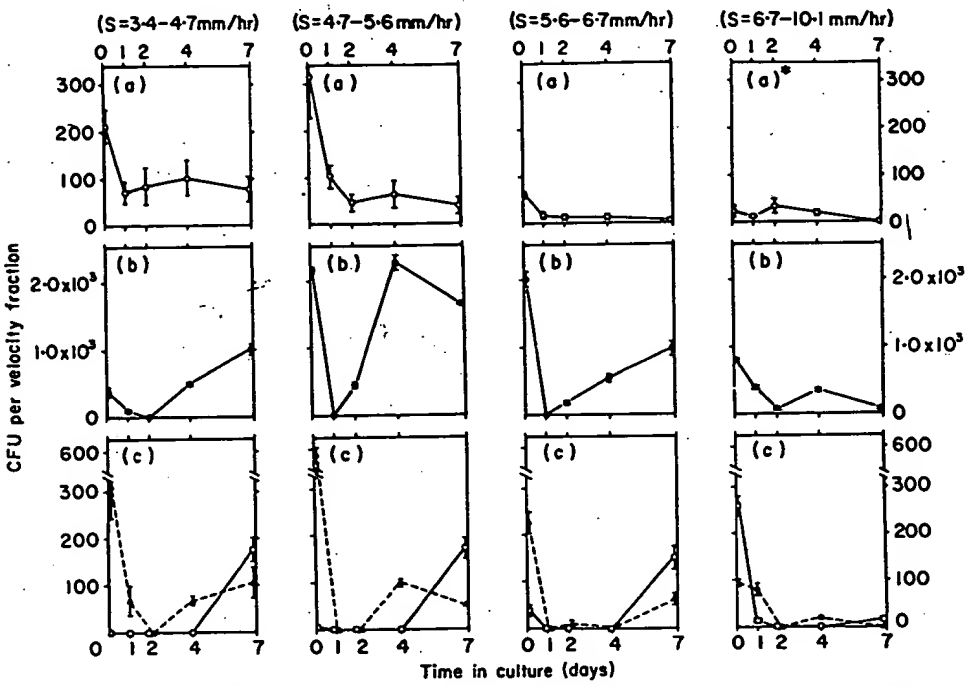

FIG. 6. Kinetics of CFU in diffusion chambers in non-irradiated mice. Normal human low density bone marrow cells were separated by velocity sedimentation. Cell fractions were pooled into four major fractions, each represented by one vertical column in the figure. S indicates sedimentation velocity. Aliquots from each pool were inoculated into fibrin-free diffusion chambers and cultured i.p. in non-irradiated mice. The number of CFU per fraction was measured before (day 0) and at various time intervals post implantation. All (a) sections of each column: neutrophilic CFU-d. All (b) sections: cells capable of cluster formation day 7 in agar. All (c) sections: day 14 (▲) and day 7 (□) colony-forming cells in agar culture. During the conditions of the experiment, no DNA-synthesis of CFU-c was detectable. All results mean ±1 s.e.m. * Neutrophilic colonies.

Each vertical column in Fig. 6 represents the results obtained with one fraction pool. The number of CFU measured before culture is indicated to the left in each column. Neutrophilic CFU-d decreased initially during the first 1 to 2 days of culture, as shown in the top sections of Fig. 6. After that time, no significant change in their number was observed. Eosinophilic CFU-d increased slighly in culture in all four fraction pools (data not shown). CFU-c and cluster-forming cells in agar disappeared completely within 1 to 2 days, but subsequently reappeared in the cultures. Day 7 cluster-forming cells (middle sections) and day 14 CFU-c (lower sections) reappeared simultaneously between day 2 and 4 of culture. Cells capable of colony formation on day 7 in agar returned later, i.e. after day 4 of culture in diffusion

chambers. The observed increases in CFU-c were most pronounced in fractions of slowly sedimenting cells, as already described. Three other experiments were carried out using cells that sedimented at between 4·5 and 5·6 mm/hr by velocity separation as the culture inoculum. The initial decrease in CFU-c did not always result in a complete disappearance of the cells from the diffusion chambers as in the experiment illustrated. However, the sequences of reappearance of cells capable of forming clusters and colonies in agar were highly reproducible. When the cells were precultured in suspension cultures (five experiments) or in diffusion chambers in irradiated, rather than in non-irradiated mice (four experiments), the same sequences were observed. In no cases was an increase in neutrophilic CFU-d in fractions initially rich in CFU-c observed.

CFU-c harvested from cultures formed colonies which were not morphologically different from agar colonies formed by cells in freshly aspirated marrow. Less than 15% of agar colonies were eosinophilic colonies. Day 7 colonies were mainly composed of polymorphonuclear neutrophils. In day 14 colonies large numbers of mononuclear cells and macrophages were found in addition to the granulocytes.

DISCUSSION

Normal neutrophilic CFU-d decreased in number in suspension cultures *in vitro* (Fig. 2) and in diffusion chambers implanted into non-irradiated mice (Figs 3 and 6). In chambers implanted into irradiated mice (Fig. 3) the number of neutrophilic CFU-d increased to a maximum on day 2 and subsequently decreased, as previously reported (Jacobsen, 1977). In contrast, CFU-c increased in number between days 2 and 7 in all culture systems employed (Figs 2, 3 and 6). Similar increases have been described by several groups (Iscove *et al.*, 1972; Hoelzer *et al.*, 1976). Provided that the observed changes in numbers of CFU were not due to changes in populations of interacting cells that stimulated or inhibited colony formation in the assays, these data supported the contention that colonies in diffusion chambers and in agar cultures were derived from different cell types.

It is possible that the observed changes in the number of CFU were not only influenced by cell proliferation, recruitment, differentiation and death during culture, but also resulted from technical artefacts, such as cell loss during harvesting of cells from the cultures. This component may be particularly important regarding diffusion chamber cultures in non-irradiated mice. The maximum decrease in neutrophilic CFU-d in these cultures was observed between day 0 and days 1–2 (Figs 3 and 6). As illustrated in Fig. 1, the day 0 value was obtained by fibrin clot diffusion chamber culture of cells which were not harvested from diffusion chambers before the assay. Therefore, the initial decrease may in part reflect cell loss during the harvesting procedure. On the other hand, separate experiments have shown that such cell loss is usually less than 20% (Jacobsen, unpublished results).

Host mouse irradiation exerts an enhancing effect on neutrophil granulocyte production by normal bone marrow cells in diffusion chambers (Böyum *et al.*, 1972; Jacobsen & Fauerholdt, 1976). As seen in Fig. 3, the number of neutrophilic CFU-d and CFU-c which could be recovered from the diffusion chambers was increased when the mice were irradiated prior to chamber implantation. The results presented in Table 1 suggested that CFU-c were proliferating in cultures in irradiated mice, but were mainly in a non-cycling state in cultures implanted into non-irradiated mice. A similar effect of host irradiation on neutrophilic CFU-d has previously been reported (Jacobsen, 1977).

Fig. 2 illustrates that eosinophilic and neutrophilic CFU-d had different kinetics in culture. The same difference was observed in diffusion chamber cultures in non-irradiated mice (data not shown). Eosinophilic granulocyte production in diffusion chambers is not significantly enhanced by host irradiation (Jacobsen & Fauerholdt, 1976), and eosinophilic CFU-d proliferate both in irradiated and in non-irradiated host mice (Jacobsen, 1977). Since the number of eosinophil colonies in diffusion chambers is frequently very low, the present report has been dealing mainly with neutrophilic CFU-d. The vast majority of colonies formed by CFU-c in agar were composed of neutrophils and/or monocyte–macrophages.

Sutherland *et al.* (1971) have demonstrated that CFU-c harvested from mouse bone marrow suspension cultures were derived from a population of cells which sedimented more slowly than the vast majority of CFU-c, but had a similar sedimentation velocity to multipotent stem cells (CFU-s), identified by the spleen colony assay in mice. Iscove *et al.* (1972) demonstrated the existence of a similar population of cells in normal human marrow, physically separable from CFU-c and capable of giving rise to CFU-c in suspension culture. These authors utilized a 14 day methyl cellulose assay system for CFU-c. The experiments illustrated in Fig. 4 showed that CFU-c, harvested after 7 days in suspension culture or diffusion chambers, were derived from cells which had the same sedimentation velocity profile as CFU-D. There was no increase in CFU-d in fractions which initially contained peak numbers of CFU-c.

Analysis of CFU-c populations in suspension cultures of separated velocity fractions (Fig. 5) showed that day 7 CFU-c harvested after 3 days in culture were derived from cells that had similar velocity sedimentation profiles to day 14 CFU-c (peak at 5·6 mm/hr). These experiments also suggested that cells which sedimented more slowly than CFU-c by velocity sedimentation (i.e. peak at 4·7 mm/hr) gave rise to day 14 CFU-c before they gave rise to day 7 CFU-c. The same sequence of appearance of CFU-c populations was observed in diffusion chambers *in vivo* (Fig. 6).

Previously published velocity sedimentation experiments have suggested that cells capable of forming clusters on day 7 in agar were a heterogeneous population of cells, which on an average sedimented more slowly than day 7 CFU-c (Jacobsen *et al.*, 1978). One subpopulation was found in the same velocity fractions as day 14 CFU-c, suggesting that some day 7 cluster-forming cells may be identical with day 14 CFU-c. This contention was supported by the results presented in Fig. 6, which showed that these progenitors had similar kinetics in culture. Similarity between the kinetics of day 14 CFU-c and a subpopulation of day 7 cluster-forming cells was also observed in the experiment presented in Fig. 5 (data not shown).

The contention that CFU-c were recruited from more immature cells in culture was strongly supported by the observation that their number increased markedly during experimental conditions where no significant DNA synthesis, i.e. cell proliferation, was observed (Fig. 6). The results reported here are compatible with the hypothesis that CFU-d give rise to CFU-c in suspension culture and diffusion chambers. The first detectable progeny of CFU-d may be cells capable of forming clusters on day 7 and colonies on day 14 in agar. This pattern of growth in agar may suggest low responsiveness of day 14 CFU-c to CSA derived from leucocyte feeder layers. Subsequent maturation of progenitor cells in diffusion chambers or suspension cultures may then result in formation of day 7 CFU-c, capable of responding to CSA by rapid proliferation in agar cultures.

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